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Protein Synthesis by Intact *Coxiella burnetii* Cells

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Coxiella burnetii was isolated from persistently infected fibroblast host cells by a rapid mechanical lysis technique. Macromolecular synthesis was initiated in these otherwise dormant cells by incubation at pH 4.5. The synthesis of protein proceeded for as long as 24 h. Initiation of protein synthesis in *C. burnetii* was dependent upon RNA synthesis. Approximately 24 species of polypeptides were synthesized, and some of these appeared to be major synthetic products. Increases in protein biomass of 15 to 30% were calculated to occur during incubation. Inhibition of DNA synthesis affected protein synthesis after 12 h of incubation. The results suggest that although these parasitic bacteria did not grow in the axenic media devised, significant biosynthetic processes occurred.

Coxiella burnetii is an intracellular parasite and the etiological agent of Q fever. *C. burnetii* grows within the phagolysosome (2), which subjects it to an acid pH of 4 to 5 (4). The acidophilic nature of this organism has been demonstrated through the stimulation of nutrient transport and catabolism in intact cells at pH 4.5 (6, 7). Furthermore, prolonged maintenance of the adenylate energy charge of *C. burnetii* was possible at either pH 4.5 or 7.0 if the medium was supplemented with the appropriate substrate, notably glutamate or pyruvate (8). Biosynthetic pathways were also operative in those studies because radiolabeled glucose, glutamate, thymidine, uridine, and guanosine were incorporated into acid-insoluble material at pH 4.5 (6). However, few details were known about macromolecular synthetic processes in those intact *C. burnetii* cells during incubation in the axenic media studied.

The *C. burnetii* genome size is 1.04×10^9 daltons (15), which provides a coding capacity for a large number of polypeptides. Therefore, it was expected that determination of the number of polypeptide species synthesized by the organism during incubations in axenic media might indicate either a complete growth cycle or a maintenance state. Furthermore, the determination of requirements for protein synthesis by *C. burnetii* in axenic media could provide useful information on the initial physiological processes occurring during infection. In studies on in vitro protein synthesis with cell-free extracts of *C. burnetii*, Donahue and Thompson (5) observed a noticeable lack of endogenous mRNA activity. These workers postulated that the rickettsial mRNA was quite labile. Hence, de novo mRNA synthesis would be a prerequisite for de novo protein synthesis in isolated *C. burnetii* cells and therefore may characterize an early step in the infection process.

Maintenance of a high energy charge in isolated *C. burnetii* cells could provide a better opportunity to obtain axenic growth (8). Classic methods of propagation of *C. burnetii* in yolk sacs of embryonated hen eggs afford luxuriant growth of rickettsiae. However, the purification protocol is lengthy and cumbersome and may not be fully suitable for these metabolic studies. Therefore, we developed a more convenient method for the culture and isolation of *C. burnetii* applicable for studies on macromolecular synthesis.

This communication confirms earlier observations concerning the acidophilic nature of *C. burnetii* (6–8). Studies performed on rickettsiae derived from heavily infected baby hamster kidney (BHK-21) cells, released either naturally or through mechanical lysis, indicate that moderate rates of protein biosynthesis occur in this organism when incubated in an axenic medium at pH 4.5. The results are discussed relative to the infection process of *C. burnetii* as it is believed to occur in vivo.

MATERIALS AND METHODS

Growth of BHK cells. BHK-21 cells, clone 13, were grown at 34°C in Dulbecco modified Eagle medium (DME; Flow Laboratories) containing 0.37% sodium bicarbonate (gas phase, 19% CO_2, 81% air) and 10% heat-inactivated calf serum (Flow Laboratories). The viability of BHK cells was determined by the trypan blue exclusion method (16). No mycoplasma contamination was detected throughout these experiments.

Infection of BHK cells with *C. burnetii*. The Nine Mile strain of *C. burnetii* (which had previously been passed three times in male Hartley guinea pigs) was used for all experiments. Confluent BHK cell monolayers (ca. 3×10^5 cells per cm^2) were washed twice with Dulbecco phosphate-buffered saline (PBS) and

then overlaid with 0.30 ml (per 75-cm^2 flask) of a 10^{-1} dilution of homogenized infected guinea pig spleen suspended in brain heart infusion broth. This solution contained 5.7×10^2 50% tissue culture infectious dose units per ml as determined in BHK cells by the method of Reed and Muench (17). Rickettsiae were allowed to absorb at room temperature for 30 min, after which monolayers were fed with 15 ml of complete DME. BHK cells were cultivated in monolayers for approximately 20 days before a persistent infection was established. At the time these experiments were performed, infected cells had been passaged between 10 and 20 times.

Rickettsial isolation. The spent tissue culture medium was replaced with fresh medium 12 to 15 h before harvest of nearly confluent, infected BHK cells. This allowed a population of naturally released *C. burnetii* organisms to accumulate. To obtain naturally released organisms, the tissue culture medium was removed and the monolayers were washed once with DME. Rickettsiae were pelleted by centrifugation at 30,000 × *g* for 25 min at 4°C. The resulting pellets were washed twice in Buffer A (22.3 mM K_2HPO_4, 135.7 mM KCl, 13.4 mM NaCl, 89 mM glycine, 10 mM $MgCl_2$, 1 mM glucose, 1 mM glutamate, 250 mM sucrose; pH 7.0) and then stored at 4°C. Infected BHK cells were harvested by trypsinization and subsequent centrifugation at 3,000 × *g* for 10 min at 4°C. Mechanically released *C. burnetii* cells were obtained by suspension of the BHK cell pellet in 20 ml of Buffer A, followed by five vigorous vortexing cycles (1 min of vortexing followed by 30 s in an ice bath). Unbroken host cells and debris were removed by centrifugation at 3,000 × *g* for 10 min. The resulting supernatant solution was saved at 4°C, and the pellet was subjected to a second vortex-lysing–centrifugation cycle in 10 ml of Buffer A. The combined supernatant solutions were centrifuged at 30,000 × *g* for 25 min at 4°C, and the pellets were washed once. The yield of *C. burnetii* was approximately 10^9 naturally released cells and 3×10^9 mechanically released cells per 75-cm^2 flask.

Incubation for macromolecular synthesis. The final pellets of naturally and mechanically released *C. burnetii* cells were suspended in Buffer B (49 mM KH_2PO_4 or K_2HPO_4, 169 mM KCl, 16 mM NaCl, 120 mM glycine, 250 mM sucrose; pH 4.5 or 7.0) and immediately added to an axenic medium (7) with 0.1 mM glutamate and 0.1 mM glucose and supplemented with the remaining 18 amino acids at a final concentration of 60 μM unless otherwise indicated. *C. burnetii* organisms were at a final concentration of 0.5×10^9 to 3.0×10^9 cells per ml in this medium.

Incorporation of radioactivity was determined by spotting 0.1 ml of the rickettsial suspension onto Whatman no. 3 filter disks for each data point. Filters were air dried and then processed by the hot trichloroacetic acid method of Mans and Novelli (12). Filters were assayed with 5 ml of a toluene-based scintillation cocktail in a Packard 460C Tricarb scintillation counter.

C. burnetii organisms were counted by the method of Silverman et al. (18). However, a double fluorescent staining method was employed whereby the *Shigella dysenteriae* population was stained in 3 mg of ethidium bromide per ml for 24 h, washed twice in PBS, and then counted in a Petroff-Hausser chamber. The standardized *S. dysenteriae* solution was then mixed with Formalin-treated *C. burnetii* cells, which were stained by the method of Chen (3). The resulting mixture was immediately sprayed onto ethanol-washed microscope slides, and the concentration of *C. burnetii* was determined as described (18).

Extraction of protein and gel electrophoresis. Upon completion of incubations, rickettsial suspensions were first chilled and then pelleted for 60 min at 30,000 × *g* at 4°C. The pellets were suspended in a solution of 10 mM Tris (pH 7.4), 1 mM $MgCl_2$, 1 mg of lysozyme per ml, and 12 μg of DNase per ml and subjected to five cycles of freeze-thawing in dry ice-acetone and 37°C, respectively. Nonidet P-40 (0.6%) was added, and the protein was solubilized at room temperature for 1 h and then frozen at −20°C until electrophoresis could be performed. Electrophoresis was performed by the method of Laemmli (10) in 8 to 15% linear gradient polyacrylamide gels (with respect to the acrylamide concentration). Proteins were stained and fixed as described (10), and radioactive polypeptides were visualized through fluorography by the method of Bonner and Laskey (1) or with En^{3}Hance (New England Nuclear Corp.). Density of polypeptide bands appearing in fluorographs was determined in a Beckman DU-8 spectrophotometer with attached gel scanning unit.

RESULTS

Isolation of *C. burnetii* from infected BHK cells. When BHK cell cultures were challenged with *C. burnetii*, up to 95% of the cells eventually became infected. Many of the infected cells contained large vacuoles which resulted in gross deformity of the typical fibroblast shape. Greater than 90% of the BHK cells were lysed by the procedure used to obtain *C. burnetii* from the intracellular environment. *C. burnetii* cells which escaped host cell confinement and had accumulated in the medium over a 12- to 15-h period were also obtained. These naturally released cells represent an extracellular transit stage of the microbe's life cycle, because they are both infectious and biochemically active.

Amino acid incorporation and effects of inhibitors. Tissue culture-grown *C. burnetii* demonstrated significant incorporation of [^{3}H]leucine into hot (90°C) trichloroacetic acid-precipitable material when incubated in a defined medium at pH 4.5. However, *C. burnetii* incubated at pH 7.0 under otherwise identical conditions showed no significant [^{3}H]leucine incorporation (Fig. 1). Both infection-released and mechanically released *C. burnetii* cells demonstrated the same rate of leucine incorporation (0.11 nmol per 10^9 cells per h) during the first 4 h of incubation. Both populations then slowed to a rate of 0.05 nmol per 10^9 cells per h (Fig. 1A). Thereafter, the protein synthetic rate in mechanically released *C. burnetii* cells decreased gradually, and after 10 h of incubation no further significant leucine incorporation was observed (Fig. 1B). When the leucine concentration in the medium

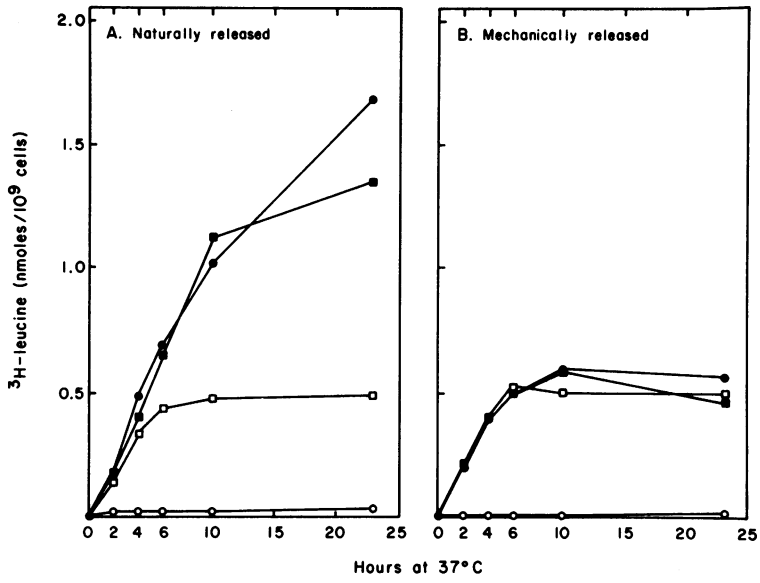

FIG. 1. Leucine incorporation by *C. burnetii*. *C. burnetii* cells were isolated and incubated with [^{3}H]leucine (20 μCi/ml, 0.33 Ci/mmol) as described in the text. Incorporation by naturally released cells (A) or mechanically released cells (B) was measured at pH 4.5 (●) and in the presence of 1 μM atractyloside (■) or 1 mM hydroxyurea (□). Incorporation at pH 4.5 in the presence of 150 μM chloramphenicol or 40 μM rifamycin, or at pH 7.0, is represented by a single line (○). The results are of a single typical experiment which has been repeated at least three times.

was lowered from 60 to 6 μM, there was a decrease in the amount of [^{3}H]leucine incorporated (not shown). At the lower concentration, naturally released *C. burnetii* incorporated leucine (exogenously added) at a rate of 0.03 nmol per 10^9 cells per h during the first 6 h of incubation at pH 4.5. Under these same conditions, mechanically released cells incorporated 0.02 nmol per 10^9 cells per h during the first 4 h of incubation at pH 4.5, after which incorporation ceased. Incorporation of [^{35}S]methionine proceeded at rates similar to those of leucine in both mechanically released and naturally released cells at pH 4.5; incorporation of methionine was not observed at pH 7.0 in either cell type. After 24 h of incubation, the pH of these media remained unchanged.

The involvement of protein and nucleic acid synthesis in [^{3}H]leucine incorporation into hot trichloroacetic acid-precipitable material was studied with several inhibitors of macromolecular synthesis (Fig. 1). Chloramphenicol and rifamycin caused nearly complete inhibition of leucine incorporation. Hydroxyurea caused significant inhibition of overall [^{3}H]leucine incorporation in naturally released *C. burnetii*, and it produced incorporation kinetics quite similar to identically treated, as well as untreated, mechanically released cells. Atractyloside demonstrated no effect on either cell type during the first 2 h of incubation.

Electrophoresis of labeled proteins. Radiolabeled *C. burnetii* proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, fluorographed, and analyzed by densitometry. Determination of protein molecular weights was based on comparison with known standards on the same gels. The molecular weight values for the proteins indicated in Fig. 2 are mean values obtained from several gels. Qualitatively the two cell types appeared similar in pattern. Approximately 24 polypeptides were synthesized at pH 4.5 by each type. However, two polypeptides (with molecular weights of 87,000 and 27,000) were radiolabeled by naturally released *C. burnetii* cells, but were not significantly labeled by mechanically released organisms (Fig. 2A). A 62,000-dalton polypeptide was radiolabeled by mechanically released *C. burnetii*, but was not significantly labeled by naturally released cells (Fig. 2B). Similar results were obtained when [^{3}H]leucine was substituted for [^{35}S]methionine.

Uridine incorporation by *C. burnetii*. Significant incorporation of [^{14}C]uridine into cold (4°C) trichloroacetic acid-insoluble material was observed in mechanically released *C. burnetii* when incubated at pH 4.5 (Table 1). However, incorporation of [^{14}C]uridine by naturally released cells under identical conditions was low at either pH 4.5 or pH 7.0 (Table 1). The incorporation of uridine by mechanically re-

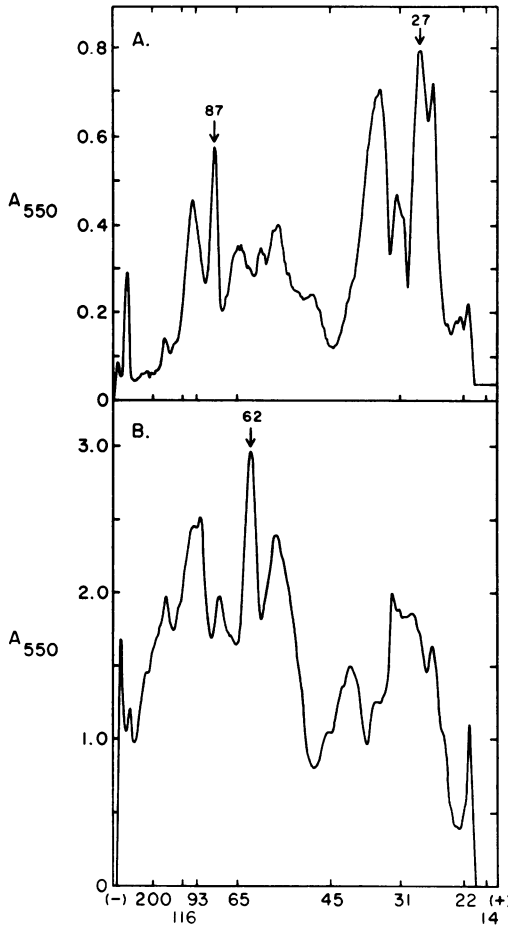

FIG. 2. Analysis of polypeptides synthesized by *C. burnetii*. *C. burnetii* cells were harvested after axenic incubation for 25 h at pH 4.5 in the presence of [^{35}S]methionine (27.3 μCi/ml, 4.53 Ci/mmol). Proteins were solubilized and electrophoresed as described, and fluorography was performed. Densitometry tracing of fluorographs of naturally released cells (A) and mechanically released cells (B). The scales used correct for different amounts of sample radioactivity applied to the gel.

leased cells proceeded for at least 6 h and was inhibited by rifamycin and actinomycin D (Table 1). About 90% of the total uridine incorporation in the presence of chloramphenicol occurred in the first 2 h of incubation. This suggested that RNA polymerase activity in the mechanically released cells was preexisting and capable of surviving the procedure used to isolate this population of *C. burnetii* from host cells. It also indicated that some protein synthesis must occur to have maximal and sustained RNA synthesis.

Effects of isolation method on amino acid incorporation into protein. To determine whether the difference in extent of protein synthesis by naturally released and mechanically released cells (Fig. 1) was due to the more rigorous method of isolation used to obtain the mechanically released population, the following experiment was performed. Naturally released cells were harvested from the infected tissue culture medium by centrifugation as usual. The pellet was suspended in 20 ml of Buffer A and added to intact infected BHK cells, and the protocol used to isolate mechanically released *C. burnetii* was performed. The incorporation of [^{3}H]leucine into hot trichloroacetic acid-insoluble material at pH 4.5 by the resulting mixture of mechanically released and naturally released cells was determined. The sum of incorporation at 24 h by equivalent amounts of naturally released cells (1.18 nmol per 10^9 cells) and mechanically released cells (0.52 nmol per 10^9 cells), incubated alone, equaled that of the mixture of the two cell types (1.74 nmol per 10^9 cells). Therefore, mechanically released *C. burnetii* cells did not appear to be injured in amino acid incorporation as a result of the isolation technique.

The effect of the tissue culture medium on the ability of *C. burnetii* to incorporate amino acids was also investigated. Mechanically released *C. burnetii* cells were preincubated at approximately 5×10^8 cells per ml at neutral pH under the conditions described in Table 2. The bacteria were then harvested, and their capability to incorporate [^{3}H]leucine into protein at pH 4.5 was measured. When cells were preincubated in either PBS or DME without calf serum, subsequent activity decreased greater than 90% (Table 2). Conversely, if mechanically released cells were preincubated in either solution with 10% calf serum, the extent of incorporation was significantly enhanced (Table 2). These results

TABLE 1. RNA synthesis by *C. burnetii*

Cell release method	pH of incubation	Antibiotic[a] treatment	Incorporation[b] (nmol per 10^9 cells)
Mechanical	4.5	None	0.270
		Rif	0.025
		Act D	0.037
		Cam	0.120
	7.0	None	0.035
Natural	4.5	None	0.059
	7.0	None	0.084

[a] Antibiotics used were 40 μM rifamycin (Rif), 10 μM actinomycin D (Act D), and 150 μM chloramphenicol (Cam).

[b] Cells were labeled with [^{14}C]uridine (1 μCi/ml, 0.463 Ci/mmol). Values indicate incorporation achieved after 24 h of incubation. Zero-time controls for incorporation ranged from 0.020 to 0.043 nmol per 10^9 cells.

TABLE 2. Effect of preincubation[a]

Preincubation medium	Incorporation (nmol of leucine per 10^9 cells) at time:		
	0 h	6 h	26.5 h
Control	0.003	0.318	0.525
PBS	<0.001	0.026	0.050
PBS + 10% calf serum	0.001	0.273	0.968
DME	0.002	0.009	0.014
DME + 10% calf serum	0.001	0.220	0.906

[a] Mechanically released *C. burnetii* cells were isolated as described in the text. The control was subjected to acid activation without prior incubation, and incorporation of [^{3}H]leucine into acid-insoluble material was determined. The remaining cells were suspended in PBS and diluted 10^{-1} in the preincubation medium indicated. Suspensions were then incubated for 19 h at 34°C in 19% CO_2. Cells were harvested and washed in a manner identical to that used for preparation of naturally released *C. burnetii*; the cells were then incubated in axenic medium in the presence of [^{3}H]leucine (50 μCi/ml, 0.83 Ci/mmol).

suggest that the enhancement effect is somehow related to the presence of calf serum and not related to the defined components of the DME.

DISCUSSION

Both types of *C. burnetii* incorporate amino acids by a pH-dependent mechanism. Inhibition of protein synthesis by chloramphenicol and rifamycin and lack of inhibition by atractyloside indicated that this incorporation is not due to host cell constituents. Lack of incorporation into mitochondria and similarly extracted uninfected cells incubated under standard conditions provided further evidence for the rickettsial nature of this activity.

Mechanically released cells incorporated significant levels of uridine at pH 4.5, whereas naturally released cells did not. Since rifamycin inhibited all protein synthesis in both cell populations, it is probable that uridine incorporation did not truly reflect the relative extent of RNA synthesis in these two cell types. Further studies are needed to determine the nature of these differences. Inhibition of protein synthesis by rifamycin also indicated that host-free *C. burnetii* cells lack viable mRNA. This agrees with the findings of Donahue and Thompson (5) and supports their hypothesis that de novo RNA synthesis must precede de novo protein synthesis in *C. burnetii* incubated in an axenic medium. Furthermore, since chloramphenicol partially inhibited RNA synthesis, it is possible that proteins involved in transcription were synthesized during incubation. Inhibition of DNA synthesis, at least in naturally released *C. burnetii*, led to an eventual decrease in protein synthesis, similar to what is observed in hydroxyurea-treated *Escherichia coli* (14). Further investigation is required to determine whether this effect is due to inhibition of DNA replication or repair.

Previous studies of axenic protein synthesis by *C. burnetii* reported incorporation rates for glutamate (6, 7), which is a preferred energy source for this organism (8) and is presumably a key intermediate in amino acid biosynthesis. To best approximate the protein synthesis rate, this study utilized leucine and methionine. These amino acids gave comparable rates and extents of incorporation when used at similar concentrations.

It was originally postulated that viable *C. burnetii* cells rapidly isolated from the phagolysosome would be best suited for studies of host cell-free protein synthesis. As the results indicate, this may not be the case. Naturally released *C. burnetii* cells demonstrated more sustained protein synthesis than their counterparts isolated from the intracellular environment. If most *C. burnetii* organisms in a given population (i.e., mechanically released or naturally released) are synthesizing protein at approximately the same rate, and given their dry weight of 27 μg per 10^9 cells (20), there was a 5 to 10% increase in mechanically released *C. burnetii* protein during acid activation. Likewise, there appeared to be an increase of 15 to 30% in naturally released *C. burnetii* protein during acid activation. If true growth occurred under these conditions, we would expect an increase in cellular protein of at least 100% and a greater diversity of polypeptides to be expressed than those observed.

The differences in the extent of protein synthesis between naturally released and mechanically released cells may be nutritional. Evidence for this is provided by the observation that mechanically released cells exposed to calf serum subsequently demonstrated a greater degree of amino acid incorporation during acid activation. This suggests that essential nutrients may be obtained during extracellular transit. Definition of these nutrients may be important in the development of a defined medium capable of sustaining axenic growth. Alternatively, the preincubation step used for mechanically released cells may have allowed the bacteria to differentiate to a sporelike quiescent state (9, 13), thus rendering them more responsive to appropriate growth conditions. However, differentiating bacterial systems often demonstrate temporal changes in the patterns of proteins synthesized (11, 19), and such differences were not readily apparent between these two cell types.

Determination of the cellular location of the proteins synthesized in axenic media, the regulation of their expression, and the demonstration of their expression in situ is needed. This may

provide a clearer understanding of the processes which occur during acid activation.

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LITERATURE CITED

1. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
2. **Burton, P. R., J. Stueckemann, R. M. Welsh, and D. Paretsky.** 1978. Some ultrastructural effects of persistent infections by the rickettsia *Coxiella burnetii* in mouse L cells and green monkey kidney (Vero) cells. Infect. Immun. **21**:556–566.
3. **Chen, T. R.** 1977. In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 Stain. Exp. Cell. Res. **104**:255–262.
4. **De Duve, C.** 1978. An integrated view of lysosome function, p. 25–38. *In* R. D. Berlin, H. Herrmann, I. H. Lepow, and J. M. Tanzer (ed.), Molecular basis of biological degradative processes. Academic Press, Inc., New York.
5. **Donahue, J. P., and H. A. Thompson.** 1980. Protein synthesis in cell-free extracts of *Coxiella burnetii*. J. Gen. Microbiol. **121**:293–302.
6. **Hackstadt, T., and J. C. Williams.** 1981. Incorporation of macromolecular precursors by *Coxiella burnetii* in an axenic medium, p. 431–440. *In* W. Burgdorfer and R. L. Anacker (ed.), Rickettsiae and rickettsial diseases. Academic Press, Inc., New York.
7. **Hackstadt, T., and J. C. Williams.** 1981. Biochemical stratgem for obligate parasitism of eukaryotic cells by *Coxiella burnetii*. Proc. Natl. Acad. Sci. U.S.A. **78**:3240–3244.
8. **Hackstadt, T., and J. C. Williams.** 1981. Stability of the adenosine 5'-triphosphate pool in *Coxiella burnetii*: influence of pH and substrate. J. Bacteriol. **148**:419–425.
9. **Khavkin, T., V. Sukhinin, and N. Amosenkova.** 1981. Host-parasite interaction and development of infraforms in chicken embryos infected with *Coxiella burnetii* via the yolk sac. Infect. Immun. **32**:1281–1291.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **27**:680–685.
11. **Linn, T., and R. Losick.** 1976. The program of protein synthesis during sporulation in *Bacillus subtilis*. Cell **8**:102–114.
12. **Mans, R. J., and G. D. Novelli.** 1960. A convenient rapid and sensitive method of measuring the incorporation of radioactive amino acids into protein. Biochem. Biophys. Res. Commun. **3**:540–543.
13. **McCaul, T. F., and J. C. Williams.** 1981. Developmental cycle of *Coxiella burnetii*: structure and morphogenesis of vegetative and sporogenic differentiations. J. Bacteriol. **147**:1063–1076.
14. **Medelson, N. H., and J. D. Gross.** 1967. Characterization of a temperature-sensitive mutant of *Bacillus subtilis* defective in deoxyribonucleic acid replication. J. Bacteriol. **94**:1603–1608.
15. **Myers, W. F., O. G. Baca, and C. L. Wisseman, Jr.** 1980. Genome size of the rickettsia *Coxiella burnetii*. J. Bacteriol. **144**:460–461.
16. **Patterson, M. K., Jr.** 1979. Measurement of growth and viability of cells in culture. Methods Enzymol. **58**:141–152.
17. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**:493–497.
18. **Silverman, D. J., P. Fiset, and C. L. Wisseman, Jr.** 1979. Simple, differential staining technique for enumerating rickettsiae in yolk sac, tissue culture extracts, or purified suspensions. J. Clin. Microbiol. **9**:437–440.
19. **Torriani, A., and C. Levinthal.** 1967. Ordered synthesis or proteins during outgrowth of spores of *Bacillus cereus*. J. Bacteriol. **94**:176–183.
20. **Williams, J. C., M. G. Peacock, and T. F. McCaul.** 1981. Immunological and biological characterization of *Coxiella burnetii*, phases I and II, separated from host components. Infect. Immun. **32**:840–851.